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Insect-Associated Bacterial Communities in an Alpine Stream

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31 **Abstract**

32 The roles of macroinvertebrate and microbial communities in stream ecosystems are recognized
33 to be important to energy flow and nutrient cycling. While the linkages of these major groups of
34 aquatic organisms has not been thoroughly investigated, determining how they interact is
35 particularly important for understanding the mechanisms and potential evolutionary relationships
36 that contribute to ecosystem processes, such as organic matter decomposition. We evaluated the
37 microbiomes of several aquatic macroinvertebrate species differing in trophic ecology and
38 belonging to different functional feeding groups at two sites along an Italian Alpine river with
39 different elevation and environmental characteristics, one located above the tree-line and the
40 other in a forested environment. We found that the internal microbial communities of the
41 different insect species significantly varied in taxonomic and functional composition and could
42 be used to classify samples to both species and environment. We demonstrated that functional
43 differences existed between the microbiota of different macroinvertebrate species with variable
44 feeding behaviors, and that species differences were more important, in this context, than
45 environmental or habitat conditions. These results provide new information on how the
46 microbiomes of macroinvertebrates may potentially be influenced by their hosts and habitat
47 conditions in Alpine streams.

48

49 **Introduction**

50 There has been limited study of entire microbial communities associated with aquatic
51 macroinvertebrates, and this is especially true in high altitude and high gradient mountain streams.
52 Evidence from other systems suggests that the internal microbial communities, or microbiota, of
53 insects and other invertebrates have important functional effects on both their biology and ecology
54 (Douglas 2015; Henry et al., 2015; Moran & Telang 1998). These studies also show that the
55 internal microbiomes may have co-evolved with certain species (e.g., ants) (Hooper et al., 2012;
56 Moran & Telang 1998; Russell et al., 2009), providing important functional roles to the fitness and
57 dispersal of many species. In addition, research has shown that the microbes of decaying organic
58 matter (Benbow et al., 2019), in the form of plant (Cummins et al., 1973; Eggert & Wallace 2007;
59 Moore et al., 2004) and animal (Pechal & Benbow 2016; Pechal et al., 2013) detritus, are acquired
60 through feeding activities and may be transferred through insect developmental stages (e.g., larvae
61 to pupae to adults) (Hocking & Reimchen 2006; Pechal et al., 2019; Weatherbee et al., 2017). In
62 freshwater ecosystems microbial communities contribute to the decomposition of autochthonous
63 and allochthonous organic matter (Baldy et al., 1995; Webster & Benfield 1986), and are known
64 to vary along the watershed continuum (Savio et al., 2015), likely responding to riparian forest
65 conditions, hydrological regimes and biotic interactions (Besemer et al., 2013; Widder et al., 2014)
66 in ways that mediate the quality and quantity of organic matter that is transported downstream.
67 However, how the internal microbial communities of aquatic macroinvertebrates contribute to
68 these processes remains largely unknown.

69
70 Organic matter subsidies vary in quantity and typology along the length of watersheds
71 (Cummins 1974; Vannote et al., 1980), and are intimately linked to the structure and diversity of

72 riparian and basin vegetation. Small order streams draining forested watersheds have significantly
73 higher allochthonous organic matter inputs (i.e., litterfall) than streams draining unforested areas
74 (Golladay 1997; Tank et al., 2010). Since the formulation of the River Continuum Concept
75 (Vannote et al., 1980), stream ecology has adopted, modified and refined (Sedell et al., 1989;
76 Statzner & Higler 1985; Winterbourn et al., 1981) a theoretic framework in which terrestrial and
77 aquatic ecosystems are intrinsically linked so that biological, physical, and chemical changes can
78 be predicted along a longitudinal gradient. Under this framework, mountainous lotic systems
79 assume great interest in understanding how organic matter is processed at the upper elevations of
80 high gradient watersheds which harbor unique sets of ecological processes and specialized taxa
81 (Ward & Saltz 1994).

82
83 Mountainous, low-order streams are distinctive systems characterized by cold, highly
84 oxygenated and turbulent water, steep gradients, coarse substrata, low channel stability and limited
85 nutrient availability (Hieber et al., 2005). Among these systems, the tree line defines separate areas
86 of the riparian zone with different limiting factors for plant growth and thus species composition
87 (Figure 1A). This abrupt change in riparian conditions is important (but largely uninvestigated in
88 Alpine regions) in influencing solar radiation and quantity and quality of terrestrial organic matter
89 subsidies. For example, below the tree line most energy inputs are derived from allochthonous
90 non-living coarse particulate organic matter (CPOM), mainly terrestrial leaves (Tank et al., 2010),
91 while above catchments have scarce terrestrial vegetation, and consequently reduced input of
92 allochthonous organic matter. Aquatic macroinvertebrate communities are known to respond both
93 taxonomically and functionally to changes in allochthonous CPOM from the riparian forests
94 (Cummins & Klug 1979; Doretto et al., 2016; Merritt & Cummins 2006; Vannote et al., 1980).

95

96 One approach to investigate the linkages between allochthonous subsidies and associated
97 biotic processing has been to evaluate functional groups of aquatic macroinvertebrates that process
98 and consume organic matter differently (Cummins 2016; Cummins & Klug 1979; Merritt &
99 Cummins 2006; Straka et al., 2012). Aquatic macroinvertebrates use a range of feeding strategies
100 to obtain nutrients, and as such, display morphological and behavioral traits which can be used to
101 classify their feeding behaviors into broad groups. These groups range from shredders, which feed
102 directly on allochthonous inputs such as leaf materials (and microbes associated with these
103 resources), to scrapers, which feed predominantly on microbial biofilms present on substrates, and
104 predators. It has been shown that the gut bacterial communities of aquatic macroinvertebrates
105 change in response to different food sources, with most studies using culture-based survey
106 approaches (Kaufman et al., 2000; Lawson et al., 1984); however, more comprehensive
107 descriptions of how the entire gut microbial community, using recent genomic sequencing
108 technologies, responds to changes in allochthonous CPOM inputs has been less studied (Pechal &
109 Benbow 2016; Yun et al., 2014).

110

111 The overall goal of this study was to describe the internal microbiota of aquatic
112 macroinvertebrate species at two elevations associated with distinct environmental characteristics
113 (mainly related to elevation and the presence or absence of riparian forest cover) along an Alpine
114 stream in Italy. We predicted that the internal microbiota of aquatic insects would differ, in part
115 based on their elevational locations, with lower microbial diversity at the higher elevation site
116 receiving less diverse CPOM, but that this difference would be mediated by individual species
117 differences.

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Materials and Methods

Study Location

Aquatic insects were collected at two sampling locations along the Po River, the longest Italian lotic system, which originates from a spring below the northwest side of the Monviso mountain, in the Cottian Alps of north-western Italy (Figure 1B). Pian della Regina (Alpine prairie) was the high elevation location at 1750 m above sea level (m.a.s.l.) and above the tree line of the drainage basin. Here the stream was an open system flowing across a plain of glacial origin, characterized by extensive Alpine meadows pointed by large erratic boulders and very few, scattered *Larix decidua* Mill., 1768. Riparian vegetation was composed almost exclusively of herbaceous species, Poaceae and Ericaceae. Within stream substrata were homogeneous and composed mainly by coarse elements (approximately 50% boulders, 40 % cobbles, 10% gravel/sand) (Figure 2A). Ostana (Forest) was the downstream, lower elevation location (971 m.a.s.l.) with forested riparian zones and slopes dominated by a mixed broadleaf forest containing *Fagus sylvatica*, *Acer* sp., *Fraxinus excelsior*, and *Alnus glutinosa*. Within stream substrata was similar to the Alpine prairie location and composed mainly of coarse elements (approximately 30% boulders, 50% cobble, 20% gravel/sand) (Figure 2B). These locations were selected by *a priori* knowledge of the aquatic macroinvertebrate taxa previously reported (Doretto et al., 2017; Fenoglio et al., 2015) where we could sample species belonging to different functional groups.

Sample Collections

140 At each location, selected aquatic insect taxa were collected on a single occasion, to
141 minimize temporal variation in gut contents and environmental variables. On 23 November 2017,
142 a season in which biodiversity and invertebrate abundance are generally highest in these
143 ecosystems (Doretto et al., 2017; Fenoglio et al., 2015), benthic insects were hand collected by
144 visually searching and turning over stream substrata. Five species that belong to the four most
145 important functional feeding groups in mountain systems were collected: ~~for scrapers~~ the mayfly
146 *Epeorus alpicola* (Eaton, 1871) (Ephemeroptera: Heptageniidae) belongs to the scraper FFG, and
147 ~~for shredders~~ the crane fly *Tipula* (*Tipulidae*) *maxima* Poda, 1761 (Diptera; Tipulidae) belongs to
148 the -shredder FFG. However, we could not find the same species of predator at both locations, so
149 two species of Systellognathan Plecoptera were used for microbiome characterizations:
150 *Dictyogenus alpinus* (Pictet, 1841) (Perlodidae) for the forested location and *Perla grandis*
151 Rambur, 1842 (Perlidae) for the Alpine prairie location. No filterer species were collected at the
152 alpine prairie location, while *Hydropsyche* sp. (Trichoptera: Hydropsychidae) were collected at
153 the forested location.

154
155 All specimens were immediately preserved in 95% molecular grade ethanol within sealed
156 glass vials for laboratory identification under a stereomicroscope using regional dichotomous keys
157 (Belfiore 1983; Fochetti & Tiernod e Figueroa 2008; Moretti 1983; Rivoecchi 1984) and then
158 weighed. This preservation approach was based on our previous success with describing the
159 microbiomes of aquatic and terrestrial macroinvertebrates (Benbow et al., 2017; Pechal & Benbow
160 2016; Pechal et al., 2019; Receveur et al., 2018; Weatherbee et al., 2017). At the time of sampling,
161 physical and chemical parameters were measured using multiparametric probes (physio-chemical
162 properties [Quanta, Hydrolab] current [Mod RHCM, Idromar]) as well as organic matter and

163 nutrients according to Italian standard methods of the Agenzia per la Protezione dell'Ambiente e
164 per i servizi Tecnici - Istituto di Ricerca sulle Acque Consiglio Nazionale delle Ricerche (APAT-
165 IRSA 2003).

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166 *Nucleic Acid Isolation and Bioinformatic Data Processing*

168 To limit the influence of microbes present on the external surfaces of the insects, after
169 preservation and immediately prior to nucleic acid isolation, all samples were surface
170 decontaminated using a 10% hypochlorite wash followed by a triple rinse in sterile water as
171 previously described (Ridley et al., 2012), and as we have done in previous work with aquatic
172 macroinvertebrates (Pechal & Benbow 2016; Receveur et al., 2018). Following surface
173 decontamination, samples were homogenized using sterile pestles as previously described (Pechal
174 & Benbow 2016; Receveur et al., 2018). Briefly, DNA extraction was performed using the Blood
175 and Tissue DNA kit (Qiagen®) with the addition of lysozyme (15 mg ml⁻¹, Invitrogen) during the
176 lysis step before being quantified fluorometrically using a Qubit 2.0 (Grand Island, NY, USA) and
177 a dsDNA High Sensitivity Assay Kit (Invitrogen). All DNA preparations were stored at -20°C
178 until library preparation. Library preparation and sequencing (2 x 250 bp paired-end reads) was
179 performed by the Michigan State University Research Technology Support Facility on an Illumina
180 MiSeq platform following previously described methods (Caporaso et al., 2011). Variable region
181 4 (V4) of the 16S rRNA gene was amplified using indexed primers 515f and 806r (5'-
182 GTGCCAGCMGCCGCGGTAA -3', 5'- GGACTACHVGGGTWTCTAAT -3') as described
183 previously (Caporaso et al., 2011; Claesson et al., 2010; Kozich et al., 2013). Demultiplexing and
184 base calling were performed using Bcl2fastq (v 2.19.1, Illumina) and RTA (v 1.18.54, Illumina).

185 The raw sequencing reads were quality filtered using QIIME 2 (v 2018.11) using default
186 settings (Bolyen et al., 2018). DADA2 was used to filter samples and remove low quality reads as
187 well as chimeric sequences and other artifacts commonly present in Illumina data (Callahan et al.,
188 2016). After singletons and amplicon sequencing variants with an abundance lower than 0.0005%
189 were removed, a Naïve Bayes classifier was trained using the region amplified by the primers
190 (515f, 806r, 250 bp) and the Greengenes database (v 13.8) at a 99% confidence level before being
191 used to assign taxonomy using default settings in QIIME 2 (Bokulich et al., 2018). Reads mapped
192 to mitochondria or chloroplast were removed. A rooted phylogenetic tree, created using all
193 remaining sequence variants and default settings in QIIME 2, FastTree (v 2)(Price et al., 2010),
194 and MAFFT (v 7)(Kato & Standley 2013) was used in calculating a phylogenetic diversity metric
195 [Faith's phylogenetic diversity (Faith's PD)]. Both Faith's PD and Shannon diversity were
196 calculated using default settings in QIIME 2 (Faith & Baker 2006). To evaluate the functional
197 community differences, PICRUSt 2 (<https://github.com/picrust/picrust2>) (Langille et al., 2013)
198 was used to assign filtered sequencing reads to functional orthologs [Kyoto Encyclopedia of Genes
199 and Genomes (KEGG) orthologs (KO)] using the mp hidden-state prediction method (Louca &
200 Doebeli 2017). Sequences files for this study have been deposited in the NCBI database under the
201 accession number PRJNA547724.

202

203 *Statistical Analyses*

204 Differences in macroinvertebrate mass between sampling location met the assumptions of
205 normality and were tested with t-tests in R (v 3.5.2) (2013). Differences in the relative abundance
206 of bacterial taxa between groups at the phylum and family level were tested using Kruskal-Wallis
207 and Mann-Whitney tests in R with FDR corrections to account for multiple comparisons. To

identify taxa which were differentially abundant at the genus level and test how well a model was able to classify samples to group (species or location), a machine learning algorithm (Random Forest) was used. Rather than test for differential abundances in all genera present (> 100), only the top indicators for each comparison were evaluated to limit the potential for spurious conclusions. The importance of an indicator in a Random Forest model is determined by how much removing that taxon from a model decreases the overall accuracy. The ten most important genus level indicators (determined by mean decrease GINI score and mean decrease accuracy) used by the models to classify samples to group were tested for differences using Kruskal-Wallis and Mann-Whitney tests in R. The random forest model was implemented using default settings in the RandomForest package (1000 trees, v 4.6-14) (Liaw & Wiener 2002). Alpha diversity metrics (Faith's PD and Shannon diversity) were compared using Kruskal-Wallis tests with a FDR correction for multiple comparisons. Beta diversity and dispersion (taxonomic and functional) were compared between site and species using PERmutational Multivariate Analysis Of Variance (PERMANOVA, Jaccard distance, 999 iterations) tests implemented in the vegan package (v 2.5-4) (Oksanen et al., 2015). Differences in beta diversity were visualized using Principle Coordinate Analysis (PCoA) plots and shown with ellipses representing 95% CIs for the mean of each group. Data were visualized using a combination of ggplot2, ggpubr, and phyloseq packages (Kassambara 2017; McMurdie & Holmes 2013; Wickham 2016) with all code used in analysis available at <https://github.com/BenbowLab/AlpineStreamMicrobiome>.

Results

Stream Conditions and Macroinvertebrate Communities

The forest sampling location had a lower mean temperature (10.5 vs 12.8 °C), higher conductivity (132 vs 98 $\mu\text{S cm}^{-1}$) and lower dissolved oxygen (9.38 vs 10.2 mg L^{-1}) than the Alpine prairie location while other parameters measured were similar (Table S1). A total of 26 samples were used for sequencing analysis (Table 1). No *Hydropsyche* sp. were collected at the Alpine prairie. Only *P. grandis* was collected from the Alpine prairie location while *D. alpinus* was collected from forested location (provided in the results but no group with less than three individuals was included in statistical analyses). The average mass of *E. alpicola* was nearly double (22.7 ± 3.86 [SE] mg vs 11.6 ± 4.0 mg) at the forest location ($t = 4.22$, $P < 0.001$), while there was not a significant difference in *T. maxima* mass between locations ($t = 1.81$, $P = 0.21$; Figure 3A).

Internal Microbial communities

From the 26 samples used for sequencing, a total of 809,647 reads were obtained after filtering, representing 2,420 amplicon sequence variants. To limit bias due to differing read sizes, samples were rarefied to 3,000 reads per sample (Figure S1). The three most abundant phyla across all samples were Proteobacteria ($51.9\% \pm [SE] 4.3$), Bacteroidetes ($17.5\% \pm 2.8$), and Firmicutes ($13.3\% \pm 3.4$), representing 83% of the total communities. As no statistically significant differences in relative taxa abundance due to environment (i.e., location) were observed at the phylum or family level between samples of the same species (Kruskal-Wallis, $P > 0.05$, Figure 3B), the two locations were combined to investigate what taxonomic differences between *E. alpicola* and *T. maxima* were conserved at both sites. The relative abundances of Proteobacteria, Firmicutes, and Planctomycetes were significantly higher in *E. alpicola* than *T. maxima* while Bacteroidetes and Firmicutes were significantly more abundant in *T. maxima* (KW, $P > 0.05$,

Figure 3C, Table S2) At the family level, there were 13 bacterial families (greater than 1% of total abundance) that were differentially abundant between *E. alpicola* and *T. maxima* (KW, $P < 0.05$, Figure 3D, Figure S2, Table S3).

To identify important genera that were differentially abundant, rather than testing for differences in every genus ($N = 170$), a random forest modeling approach was used to determine the top ten indicators of each group, which would then be tested further. Modeling was able to correctly classify which location a sample came from, regardless of species, with an Out Of Bag (OOB) error rate of 3.85% at the genus level (one sample from the Alpine prairie site misclassified as forest). The ten most important genera for classification (determined by Mean Decrease Gini score and Mean Decrease Accuracy) were then tested using Kruskal-Wallis tests to determine if they were differentially abundant between locations. None of the top ten indicators using either ranking method were significantly different between locations (KW, $P > 0.05$, Table S4, Figure S3, Figure S4).

To determine if modeling could predict species, regardless of location, and identify differentially abundant genera, samples from the two locations were combined and tested similarly as above. The random forest model was able to predict species (*P. grandis* not included in model, $N = 2$) with an error rate of 8.33% (two *Hydropsyche* sp. misclassified). All of the top ten predictors using both ranking methods were significantly different among species (KW, $P < 0.001$, Figure S5, Table S5, Table S6) with multiple comparisons (Mann-Whitney, FDR correction, $P < 0.05$) shown in Figure 4.

Bacterial community diversity

Forest *E. alpicola* bacterial communities were more phylogenetically diverse ($9.05 \pm [SE]$ 0.7, Faith's PD: KW, $\chi^2 = 6$, $P = 0.014$) than *E. alpicola* from the Alpine location (4.73 ± 0.26), while Shannon diversity was not significantly different between locations (KW, $\chi^2 = 2.94$, $P = 0.086$). For *T. maxima*, diversity was not different between locations for either alpha diversity metric (KW, $P > 0.05$). Comparing species at the forest location, *T. maxima* and *Hydropsyche* sp. displayed similar levels of bacterial diversity while having significantly higher Shannon (Mann-Whitney, $P < 0.05$) and phylogenetic diversity (MW, $P < 0.05$) than *E. alpicola* (Figure 5A, Figure 5B). A similar pattern was observed at the prairie location with *T. maxima* and *D. alpinus* having significantly higher diversity than *E. alpicola* according to both metrics (MW, $P < 0.05$).

Differences in beta diversity was visualized using PCoA plots and compared with PERMANOVA tests using Jaccard distance. Species, location, as well as their interaction impacted beta diversity, with species having the strongest effect (PERMANOVA, $P < 0.01$, Table 2, Figure 5C). As the interaction between location and species significantly influenced beta diversity, the effects of species were investigated separately for each location. At both the forest (*E. Alpicola*, *T. maxima*, and *Hydropsyche* sp.) and prairie (*E. alpicola*, *T. maxima*, and *D. alpinus*) locations, all species within a location had significantly different microbial communities from each other (PERMANOVA, $P < 0.05$).

Functional community composition

Due to the reliance on high-quality gene annotations for predicting individual functional pathways, which are lacking for many poorly characterized environmental sample types (Langille et al., 2013; Radivojac et al., 2013), predicted functional differences between location and species were explored using community diversity metrics rather than individual pathway abundances.

299 While species had a significant effect (PERMANOVA, $F = 4.81$, $P > 0.001$) on functional
300 community diversity and accounted for 41% of the variation present (R^2), location did not have a
301 significant effect ($P > 0.05$, Table 3). As there was not a significant effect of location, samples
302 from both locations were combined to determine how community functional diversity differed
303 between species. When pairwise comparisons were run between insect species (*P. grandis* not
304 tested, $N = 2$), all had significantly different functional communities (PERMANOVA, $P < 0.05$,
305 Table S7).

306

307 **Discussion**

308 In this study, we examined how the internal microbiota of aquatic macroinvertebrates
309 differed among species belonging to different functional feeding groups in two Alpine stream
310 habitats with different riparian conditions. While macroinvertebrate-microbe interactions have
311 long been recognized as an essential component of understanding food web interactions in aquatic
312 systems (Cummins & Klug 1979; Kaufman et al., 2000), this study represents the first comparisons
313 among the internal microbial communities of macroinvertebrate species using high throughput
314 genomic sequencing in Alpine stream communities. Two other recent studies using high
315 throughput sequencing to compare the microbiota of aquatic macroinvertebrate functional groups
316 occurred in Midwest streams (USA) (Ayayee et al., 2018), and associated with salmon
317 decomposition (Pechal & Benbow 2016) in Alaska (USA).

318 While macroinvertebrate feeding groups differ in their predominant method of feeding, most
319 aquatic species are omnivorous and readily uptake food from a variety of sources (e.g., scrapers
320 ingesting particulate organic matter or eukaryotes during feeding on surfaces) though their ability
321 to digest certain foods can vary due to multiple factors, including differences in their internal

322 microbiota (Pechal and Benbow 2016), pH, and oxygen conditions (Cummins and Klug 1979).
323 For example, it has been shown that crane flies (shredder, Diptera: Tipulidae) require microbial
324 conditioning of leaf surfaces for development and use gut bacteria to help break down ingested
325 food (Klug & Kotarski 1980; Lawson & Klug 1989). While in terrestrial systems, the functional
326 roles of microbes are widely documented (e.g., nitrogen fixation or cellulose degradation) (Alonso-
327 Pernas et al., 2017; Alonso-Pernas et al., 2018; Ayayee et al., 2018; Gupta et al., 2012), in aquatic
328 systems comparatively little research exists, but it is hypothesized that there are similar
329 relationships (Ayayee et al., 2018). Although we chose not to examine individual functional
330 pathways due to limitations of using gene amplicon data for this purpose in understudied systems
331 (Langille 2018; Langille et al., 2013; Radivojac et al., 2013), we found that the internal bacterial
332 functional diversity was distinctly different between species, with species explaining close to half
333 (41%) of the variation present. As macroinvertebrates from different feeding groups ingest and
334 process predominantly distinct forms of organic matter (Ayayee et al., 2018; Cummins & Klug
335 1979), it would be expected that their gut community assemblages would be adapted to different
336 functional roles, similar to terrestrial insects (Larsen et al., 2016; Mason et al., 2016). While further
337 research will be needed to elucidate exactly what functional roles these bacteria play, we
338 demonstrated both structural and functional differences among microbial communities of
339 macroinvertebrate species with different feeding behaviors.

340 We observed several differentially abundant bacterial taxa and were able to successfully
341 classify internal communities to both species and site with a high degree of accuracy. At all
342 taxonomic levels (phylum, family, and genus) there were significantly different abundances
343 between species, but no significant effects of location. In contrast to previous studies (Ayayee et
344 al., 2018; Pechal & Benbow 2016), which observed that predatory feeding groups had lower

345 phylogenetic diversity than grazers/scrapers and filterers, we observed that the predatory *D.*
346 *alpinus* had higher diversity than *E. Alpicola* (scraper), regardless of location. Predators
347 (Perlodidae: *D. alpinus*) showed similar levels of bacterial diversity (Shannon and Faith's PD) to
348 species belonging to both filter (Hydropsychidae: *Hydropsyche* sp.) and shredder (Tipulidae: *T.*
349 *maxima*) groups while species ~~of~~ belonging to the scraper groups (Heptageniidae: *E. alpicola*) had
350 lower diversity than all others (then others?). The lower phylogenetic diversity of *E. alpicola*
351 microbiota at the upstream prairie site, may be related to the lower diversity of benthic
352 microorganisms that has been shown in the upper sections of Alpine rivers compared with
353 downstream reaches (Falasco & Bona 2011). These results highlight that additional studies are
354 needed to identify factors that shape macroinvertebrate gut microbial communities and how they
355 relate to ecosystem function in Alpine stream habitats.

356 While we expected the diversity of microbial communities within *T. maxima* (shredder) to be
357 higher at the forested site due to the presence of more heterogenous allochthonous and
358 autochthonous resources below Alpine tree lines (Wilhelm et al., 2015), we observed no significant
359 differences in alpha diversity or taxa composition (phylum, family or genus level), suggesting the
360 shredder species we sampled at these sites may be acquiring gut communities with limited
361 colonization from microbes associated with their food. Though location significantly impacted the
362 beta diversity of the taxonomic bacterial communities, it had a smaller effect than species. When
363 comparing bacterial community function, location no longer had significant impacts on diversity,
364 suggesting that although taxonomic differences exist between sites, the taxa present may be
365 playing similar functional roles within the insect guts, analogous to functional redundancy in other
366 systems (Rosenfeld 2002).

367 Although differences in beta diversity were observed between sites, there were surprisingly no
 368 other significant taxonomic or functional differences between site for either *E. alpicola* (scraper)
 369 or *T. maxima* (shredder). As solar radiation and the presence of riparian vegetation significantly
 370 alters the taxonomic composition and function of biofilm communities (Wagner et al., 2015;
 371 Wilhelm et al., 2015), it would be expected to see differences between species of scrapers at the
 372 two sites that use biofilms as a predominant food source, if their microbiota simply reflected their
 373 diet. That no differences in taxonomic composition or functional diversity were observed between
 374 our scraper or shredder species at the two sites suggests their gut microbiota may be a result of
 375 selective colonization by taxa and not simply a reflection of their food. This similarity of microbial
 376 communities within insect species from different locations has previously been reported and may
 377 be a result of similar nutritional components and microbial species sorting due to similarities in
 378 the gut environment (e.g. morphology, pH, and oxygen conditions) (Anderson & Cargill 1987;
 379 Ayayee et al., 2018; Pechal & Benbow 2016).

380 *Conclusions*

381 Under the theoretical framework of the River Continuum Concept (RCC) and its derivatives (Junk
 382 et al., 1989; Vannote et al., 1980; Ward & Stanford 1995) high gradient, low order mountain
 383 streams provide a unique opportunity to investigate the impact of watershed conditions on
 384 microbial community assembly within macroinvertebrate species. While the tree line represents a
 385 drastic change in riparian conditions, we observed limited effects on internal microbial community
 386 structure and function, compared to the effects of macroinvertebrate species from different feeding
 387 groups. While there were site-specific differences in taxonomic diversity, these changes were not
 388 reflected in community function suggesting although different communities are present at the two
 389 locations, they provide similar functions. The observed dissimilarities between species of

[belonging to](#) different functional feeding groups, regardless of riparian conditions, agrees with previous research in suggesting that conditions within their digestive system allows for selective colonization of microbes, with distinct functional roles, and do not simply reflect their environment/diet (Ayayee et al., 2018). That large differences in CPOM inputs (e.g., leaf material) and light conditions did not lead to differences in individual taxa or functional changes was surprising, particularly for our shredder and scraper species as leaf material and autotrophic organisms represent their predominant food source. As algal and fungal communities comprise important roles in primary production and organic matter processing in stream systems (Cummins 1974; Danger et al., 2013; Kuehn 2016) how these taxa differ between species and functional feeding guilds will require further investigation. While this study provides initial data on how species and habitat may be linked with gut bacterial communities in alpine systems, additional studies are needed to expand on this evidence and test multiple species of each functional feeding group in several catchments. Our study was limited to one species for each functional group (except for predators), making the distinction between the effect of species and functional group difficult to untangle. Larger, more comprehensive surveys and manipulation experiments are warranted to differentiate species and functional feeding group gut microbial community structure.

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413

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415

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418

419 **Figure Captions**

420

421 **Fig. 1** a) Representative photo of tree line in Ostana. b) Study drainage basin in Italy showing the
422 elevation/altitude for the higher elevation Alpine prairie location (Pian della Regina) and lower
423 elevation forest location (Ostana). Altitude is displayed in meters above sea level.

424

425 **Fig. 2** Photos showing the riparian vegetation cover of a) high elevation Alpine prairie station
426 (Pian della Regina) and b) lower elevation forested station (Ostana).

427

428 **Fig. 3** Internal bacterial communities of macroinvertebrate species: a) Differences in mass (mg)
429 for *E. alpicola* and *T. maxima* at the two sites. Significance was determined by t-tests; b) Phylum
430 level bacterial relative abundance between species; c) Differences in phylum level relative
431 bacterial abundance between *E. alpicola* and *T. maxima*. Samples from the two sites were
432 combined and only phyla with a relative abundance greater than 1% of the total relative
433 abundance are shown. Samples were compared with Kruskal-Wallis tests with FDR correction;
434 d) Differences in family level relative bacterial abundance between *E. alpicola* and *T. maxima*.
435 Samples from the two locations were combined and only families which made up greater than

3% of the total relative abundance are shown, for lower abundance families see Figure S2. Significance between species were compared with Kruskal-Wallis tests and FDR correction. Error bars are SEM.

Fig. 4 Top ten genus level predictors (determined by mean decrease GINI) for a random forest model predicting species (*P. grandis* was not included in the model, N = 2). Error bars are SEM. Significance between pairwise comparisons (Mann-Whitney, FDR correction) are denoted by lowercase letters

Fig. 5 Differences in community diversity among macroinvertebrate species a) Differences in Faith's phylogenetic diversity (Faith's PD) between species. Comparisons between species were tested with Kruskal-Wallis tests with pairwise significance (Mann-Whitney) denoted by lowercase letters; b) Shannon diversity differences among species; c) PCoA plot (Jaccard distance) showing community differences due to taxonomic composition; d) PCoA plot (Jaccard) showing predicted functional differences in beta diversity among species. Ellipses represent 95% CI for the mean of each group. Legend is given in 5C.

Table captions

Table 1. Macroinvertebrate samples used for microbial sequencing.

458 **Table 2.** Differences in taxonomic beta diversity (Jaccard, PERANOVA) among
459 macroinvertebrate species and location (*P. grandis* not included, N = 2). SS = Sums of Squares,
460 MS= Mean Squares
461

462 **Table 3.** Differences in predicted functional beta diversity (Jaccard, PERMANOVA) among
463 macroinvertebrate species and location (*P. grandis* not included, N = 2). SS = Sums of Squares,
464 MS= Mean Squares
465

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